



King's Research Portal

DOI:

[10.1016/j.jid.2017.04.026](https://doi.org/10.1016/j.jid.2017.04.026)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Law, M. H., Medland, S. E., Zhu, G., Yazar, S., Viñuela, A., Wallace, L., Shekar, S. N., Duffy, D. L., Bataille, V., Glass, D., Spector, T. D., Wood, D., Gordon, S. D., Barbour, J. M., Henders, A. K., Hewitt, A. W., Montgomery, G. W., Sturm, R. A., Mackey, D. A., ... MacGregor, S. (2017). Genome-Wide Association Reveals Pigmentation Genes Play a Role in Skin Aging. *Journal of Investigative Dermatology*, 137(9), 1887-1894.
<https://doi.org/10.1016/j.jid.2017.04.026>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Accepted Manuscript

Genome-Wide Association Reveals Pigmentation Genes Play a Role in Skin Aging

Matthew H. Law, Sarah E. Medland, Gu Zhu, Seyhan Yazar, Ana Viñuela, Leanne Wallace, Sri Niranjan Shekar, David L. Duffy, Veronique Bataille, Dan Glass, Tim D. Spector, Diane Wood, the MuTHER Consortium

PII: S0022-202X(17)31492-6

DOI: [10.1016/j.jid.2017.04.026](https://doi.org/10.1016/j.jid.2017.04.026)

Reference: JID 858

To appear in: *The Journal of Investigative Dermatology*

Received Date: 4 January 2017

Revised Date: 5 April 2017

Accepted Date: 24 April 2017

Please cite this article as: Law MH, Medland SE, Zhu G, Yazar S, Viñuela A, Wallace L, Shekar SN, Duffy DL, Bataille V, Glass D, Spector TD, Wood D, the MuTHER Consortium, Genome-Wide Association Reveals Pigmentation Genes Play a Role in Skin Aging, *The Journal of Investigative Dermatology* (2017), doi: 10.1016/j.jid.2017.04.026.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



**GENOME-WIDE ASSOCIATION REVEALS PIGMENTATION GENES PLAY A ROLE
IN SKIN AGING**

Matthew H. Law^{1*}, Sarah E. Medland¹, Gu Zhu¹, Seyhan Yazar², Ana Viñuela³, Leanne Wallace⁴, Sri Niranjan Shekar^{1,5}, David L. Duffy¹, Veronique Bataille^{3,6}, Dan Glass^{3,7}, Tim D. Spector³, Diane Wood⁸, the MuTHER Consortium⁹, Scott D. Gordon¹, Julie M. Barbour¹⁰, Anjali K. Henders¹, Alex W Hewitt^{11,12}, Grant W. Montgomery^{13,1}, Richard A. Sturm¹⁴, David A. Mackey², Adèle C. Green^{1,15}, Nicholas G. Martin¹, Stuart MacGregor¹

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia

²Centre for Ophthalmology and Visual Science, Lions Eye Institute, University of Western Australia, Perth, Western Australia, Australia.

³Department of Twin Research and Genetic Epidemiology, King's College London, London, United Kingdom

⁴Institute for Molecular Biosciences, Program in Complex Trait Genomics, University of Queensland, Brisbane, Australia

⁵LSE Health, London School of Economics and Political Science, London, United Kingdom

⁶Dermatology, West Herts NHS Trust, Hemel Hempstead, Herts, United Kingdom

⁷Department of Dermatology, Northwick Park Hospital, London, United Kingdom

⁸The Launceston Eye Institute, Launceston, Tasmania, Australia

⁹Full list of authors for group names can be found in the supplementary information

¹⁰School of Population Health, University of Western Australia, Perth, WA, Australia

GWAS of Skin Aging

¹¹Menzies Institute for Medical Research, School of Medicine, University of Tasmania, Hobart, Australia

¹²Centre for Eye Research Australia, University of Melbourne, Royal Victorian Eye and Ear Hospital, Melbourne, Victoria, Australia

¹³Molecular Biology, the University of Queensland, Brisbane, Australia.

¹⁴Dermatology Research Centre, The University of Queensland, Diamantina Institute, Translational Research Institute, Brisbane, Australia

¹⁵CRUK Manchester Institute and Institute of Inflammation and Repair, University of Manchester, England

*Corresponding author

Correspondence: matthew.law@qimrberghofer.edu.au

KEYWORDS

Skin aging

Photoaging

GWAS

Pigmentation

Gene

ABSTRACT

Loss of fine skin patterning is a sign of both aging and photoaging. Studies investigating the genetic contribution to skin patterning offer an opportunity to better understand a trait that influences both physical appearance and risk of keratinocyte skin cancer. We undertook a meta-analysis of genome-wide association studies (GWAS) of a measure of skin pattern (microtopography score) damage in 1,671 twin pairs and 1,745 singletons (N = 5,087) drawn from three independent cohorts. We identified that rs185146 near *SLC45A2* is associated with a skin aging trait ($p = 4.1 \times 10^{-9}$); to our knowledge this is previously unreported. We also confirm previously identified loci, rs12203592 near *IRF4* ($p = 8.8 \times 10^{-13}$), and rs4268748 near *MC1R* ($p = 1.2 \times 10^{-15}$). At all three loci we highlight putative functionally relevant SNPs. There are a number of red hair/low pigmentation alleles of *MC1R*; we found that together these *MC1R* alleles explained 4.1% of variance in skin pattern damage. We also show that skin aging and reported experience of sunburns was proportional to the degree of penetrance for red hair of alleles of *MC1R*. Our work has uncovered genetic contributions to skin aging and confirmed previous findings, showing that pigmentation is a critical determinate of skin aging.

GWAS of Skin Aging

ABBREVIATIONS

Beagley–Gibson 6-point rating system (BG6)

Genome-wide association study (GWAS)

Solute carrier family 45 member 2 (SLC45A2)

Melanocortin receptor 1 (MC1R)

Interferon regulatory factor 4 (IRF4)

Ultra-violet radiation (UVR)

Reactive oxygen species (ROS)

differentially expressed in FDCP 8 homolog (DEF8)

spastic paraplegia 7 (SPG7)

dysbindin (dystrobrevin binding protein 1) domain containing 1 (DBNDD1)

Linkage disequilibrium (LD)

Single nucleotide polymorphism (SNP)

INTRODUCTION

Skin aging, characterised by epidermal thinning and reduced DNA repair, results from both external and internal influences. Major extrinsic factors include exposure to ultra-violet radiation (UVR) causing photoaging, and smoking. Specifically, photoaging is typified by elastosis and damage to the complex extracellular support matrix of the dermis (for review, see (Bilac et al., 2014)). This photodamage alters mechanical properties in the skin, leading to deterioration and loss of regularity of the fine reticular patterning of the skin surface (Lavker et al., 1980), as well as wrinkles and sagging. As UVR also induces DNA damage, the degree of photoaging correlates with skin carcinogenesis (Gandini et al., 2005, Green, 1991, Holman et al., 1984). Thus heritable factors that modify skin aging, particularly photoaging, have implications for both personal appearance and risk of skin cancer.

To date, a number of GWAS have analysed various measures of photoaging, or overall skin aging. These include perceived facial age (Liu et al., 2016), facial photoaging (Le Clerc et al., 2013) and the combined impact of intrinsic and extrinsic factors on facial aging (Chang et al., 2014). Additional GWAS have been conducted on traits related to photoaging such as actinic keratosis and facial pigmented spots (Jacobs et al., 2015a, Jacobs et al., 2015b, Laville et al., 2016). Together these have identified genetic variants on chromosomes 3, 6, 16, 20 and X.

Here we report on a meta-analysis of five GWAS drawn from three independent cohorts (Methods) analysed using the 6-point Beagley and Gibson (BG6) microtopography scoring system of skin patterning regularity and complexity (Beagley and Gibson, 1980, Holman et al.,

GWAS of Skin Aging

1984)). BG6 score strongly correlates with amounts of dermal solar elastosis (Battistutta et al., 2006, Fritschi et al., 1995, Seddon et al., 1992), more so at younger ages (Hughes et al., 2012). It is correlated with self-reported sun exposure history and skin phototype, and sunscreen can protect against the age-related increase in BG6 score (Hughes et al., 2013). At age 12 BG6 is highly heritable (86%), with heritability decreasing to 62% in adults (Shekar et al., 2005), suggesting BG6 is a useful measure to explore the genetics of photoaging. Here we aim to confirm previous GWAS findings, identify additional loci and characterise the interaction of *MC1R* red hair alleles with BG6 and various sun exposure behaviours.

RESULTS

Skin patterning GWAS meta-analysis

We performed a meta-analysis of five BG6 GWAS drawn from three independent cohorts, representing 1,671 twin pairs and 1,745 singletons ($N = 5,087$; Methods; Table 1). To account for differences in age, genotyping array and phenotype a total of five BG6 GWAS (Table 1) were conducted using imputed genotype dosage scores in Merlin-offline (Abecasis et al., 2002), a method that appropriately models the family structure in related samples, including twins (Methods, Figure 1 and 2). GWAS QQ plots indicated there was no evidence for population stratification (Supplementary Figure 1). SNPs at or near *SLC45A2*, reached genome-wide significance with a measurement of skin aging, as did SNPs near *IRF4* and *MC1R* (Table 2). Full results for all SNPs with $p < 1 \times 10^{-5}$ are reported in Supplementary Table 1). Conditioning on the top SNPs did not reveal additional associations (Supplementary Methods; Supplementary

GWAS of Skin Aging

Table 2, Supplementary Figure 5).

In addition to BG6, a number of other skin aging related traits have been analysed by GWAS (Chen et al., 2014, Jacobson et al., 2015a, Jacobson et al., 2015b, Laville et al., 2016, Le Clerc et al., 2013, Liu et al., 2016). Supplementary Table 3 summarises the BG6 association for those SNPs previously reported as reaching genome-wide significance with other skin aging related traits. Of note, the top SNP from the chromosome 3 region reported in Le Clerc et al., (2013) is associated with facial photoaging, rs322458 is $p = 0.07$ for BG6; more promising is the nearby SNP rs470647, noted as a possible functional SNP tagged by rs322458 (BG6 $p = 3.8 \times 10^{-3}$).

Gene-based approach

We used the gene-based association test, VEGAS2 to test for multiple independent signals within a gene that are individually not genome-wide significant, (Mishra and Macgregor, 2015) (Supplementary Methods). All results with a p -value $< 5 \times 10^{-5}$ are reported in Supplementary Table 4. Although many genes at the 16q24.3 locus have significant gene based p -values ($p < 2.084 \times 10^{-6}$), due to extensive LD across this region, this is driven by red hair alleles in *MC1R*.

Functional annotation

As the top SNP at each locus may not be the functional one, meta-analysis results were filtered to p -values $< 1 \times 10^{-7}$ and homogenous effects sizes across contributing studies ($I^2 < 31\%$), leaving 102 SNPs mapping to the three genome-wide significant regions on chromosomes 5, 6 and 16

GWAS of Skin Aging

(Supplementary Table 5; Supplementary methods). Our annotation work did not identify any additional regulatory or functional SNPs at 5p13.2/*SLC45A2* beyond that our peak SNPs are in LD ($r^2 = 0.73$) with known missense rs16891982 (F374L) variant. The L374 allele, rare in European derived populations is associated with darker skin (Graf et al., 2005) and here with reduced BG6 score, indicating less photoaging (Table 2). The association at 6p25.3, near *IRF4* is strongest at rs12203592, which lies in a melanocyte enhancer and is a weak *IRF4* eQTL in whole blood and lymphocytes (GTEx $p = 6.0 \times 10^{-7}$ and $p = 5.0 \times 10^{-7}$). rs12203592 has been shown to modulate *IRF4* regulation (Praetorius et al., 2013). In the MuTHER dataset, 17 BG6 associated SNPs at 16q24.3 are eQTLs for 7 genes (Online methods, Supplementary Table 6) while an overlapping list of 16 SNPs are eQTLs for 10 genes in the Westra data (Westra et al., 2013) (Supplementary table 7). As reported in Grundberg et al., 2012 these include the *MC1R* red hair SNP rs1805007 as an eQTL for *DBNDD1* (Grundberg et al., 2012). Together these reveal direct impacts on pigmentation via non-synonymous changes to *SLC45A2* and *MC1R*, as well as expression modulation of *IRF4*. Annotation also highlights that *MC1R* red hair alleles may also be associated with altered expression in additional genes, which may further influence skin patterning and photoaging.

Impact of *MC1R* alleles on sun exposure effects

Our GWAS meta-analysis provides evidence that red hair alleles of *MC1R* are associated with skin patterning. 1,246 of the adolescent twins used in the adolescent Phase 1 GWAS (Table 1) have been previously genotyped for nine low frequency coding *MC1R* alleles (Duffy et al., 2004). To improve our statistical power we grouped these *MC1R* alleles by their penetrance for

GWAS of Skin Aging

red hair and tested them for association with a range of sun exposure behaviours; a summary of these measures and their correlation can be found in the supplementary methods and Supplementary tables 8 and 9. Male and female participants were analyzed separately given the influence of sex on skin patterning (Supplementary Methods).

There were significant differences in BG6 for *MC1R* compound genotypes (having any combination of two red hair alleles at *MC1R*) in both sexes ($p < 0.001$, Figure 3), with a significant trend for increasing BG6 score and greater red hair penetrance ($p < 0.001$). Together *MC1R* red hair alleles explain at least 4.12% of BG6 variation. rs12203592 in *IRF4* and rs16891982 in *SLC45A2* explain a further 2.95% and 0.26% of BG6 variation respectively. Sunburn risk increased for compound genotypes in line with the degree of penetrance for red hair (Figure 4). Additionally, we explored the impact of *MC1R* genotype and thus vulnerability to skin damage on sun exposure behaviour and utilization of sun protection (Supplementary Methods). We concluded there were no differences in Sun Exposed Hours (UV index justified, Supplementary Figure 6) or sun protection behaviour between the *MC1R* genotype groups (Supplementary Figure 7). The genome-wide significant SNP rs12203592 in *IRF4* did not exhibit any association with sun exposure or protection traits (data not shown).

DISCUSSION

We show that *SLC45A2* SNPs in LD with the non-synonymous rs16891982 (F374L; Graf *et al.*, 2005) are genome-wide significantly associated with a measure of skin aging, BG6; to our knowledge this is previously unreported. We also replicate previous associations at *IRF4* and *MC1R* (Supplementary Table 2) (Jacobset al., 2015a, Jacobset al., 2015b, Liuet al., 2016). The associations include well-known alleles affecting pigmentation (Duffyet al., 2004). For each variant, the allele correlated with greater skin pigmentation was associated with reduced photoaging. A functional SNP rs470647 previously reported to be associated with facial photoaging (Le Clercet al., 2013), was associated ($p = 3.8 \times 10^{-3}$) with BG6. The detection of overlapping SNPs using a range of different measures of skin aging is promising (Supplementary Table 3), and confirms that there are germline effects on multiple skin aging components. The combined effects of the seven *MC1R* red hair alleles polymorphic in our population explained at least 4.12% of BG6 variation, representing a gene of large effect compared to most quantitative trait loci.

Replication across independent datasets is critical for confidence in GWAS findings. Data contributing to this meta-analysis were drawn from three different Australian cohorts, and analyzed as five discrete GWAS to account for differences in genotyping array. BG6 associated SNPs at *SLC45A2* and *MC1R* were at least $p < 0.05$ in three or more GWAS sets drawn from two independent cohorts; however, SNPs at *IRF4* did not pass the imputation threshold in Raine (Supplementary Table 1). Nevertheless, the fact we replicate skin aging association at *MC1R* and *IRF4* suggests our observations are robust. While conditional analysis did not reveal additional genome-wide significant associations (Supplementary Figure 5), a second red hair allele,

GWAS of Skin Aging

rs1805008 (R160W) was also associated with BG6 at $p = 1.1 \times 10^{-5}$ (Supplementary Table 1), suggesting like many other complex traits, additional variation remains to be discovered with larger sample sizes.

The three loci associated with BG6 are well characterised, giving direct insights into how they influence fine skin patterning. Animal models suggest *SLC45A2* low activity alleles impair the processing and transport of melanogenic proteins to melanosomes (Costin et al., 2003), possibly via altered ability to maintain melanosome pH (Dooley et al., 2013). As well as regulating pigmentation together with *SLC45A2*, *MC1R* signalling enhances melanocyte UVR resistance by rapidly upregulating antioxidant defences (Song et al., 2009) independently to melanin synthesis (Maresca et al., 2010). Melanocytes can act as a sink for reactive oxygen species (ROS) generated by keratinocytes in vitro (Pelle et al., 2005), and *MC1R* red hair alleles have a reduced capacity to mediate ROS protection (Kadekaro et al., 2010). *MC1R* null ‘red hair’ mice are at higher risk of melanoma and oxidative DNA damage in the absence of UV, suggesting the increased synthesis of pheomelanin is itself pro-oxidant (Mitra et al., 2012). In parallel to animal models, there is some evidence that *MC1R* red hair alleles are associated with higher risk for keratinocyte skin cancers in a high pigmentation background (Bastiaens et al., 2001, Box et al., 2001). Skin cancer risk is likely influenced by the fact that individuals carrying *MC1R* variant genotypes experience more sunburns (Figure 4). Liu et al., 2016 reported that any influence of *MC1R* on perceived aging is independent of wrinkling; here we show that *MC1R* SNPs are associated with higher BG6 scores, which correlates with greater photoaging, dermal solar elastosis and wrinkling. This suggests that these genetic variants are not acting independently of wrinkling.

GWAS of Skin Aging

We have used VEGAS2 to identify multiple genes near *MC1R* as potentially associated with BG6 (Supplementary Table 4). *MC1R* red hair alleles, and SNPs in LD, are eQTLs for genes other than *MC1R* (Grundberg et al., 2012, Westra et al., 2013). However, it is also possible that the observation of additional genes via VEGAS2, and *MC1R* red hair alleles acting as eQTLs, are simply a product of the strong selection on the *MC1R* locus in Europeans and resulting long range LD.

Three SNPs at 6p25.3 are genome-wide significantly associated with BG6, with the lowest p -value at rs12203592 ($p = 1.0 \times 10^{-13}$) in intron four of *IRF4* (Table 2). Variants in *IRF4* including the T allele of rs12203592 (associated with higher BG6 or more severe skin aging) have been associated with lower, pigmentation (but not red hair), poorer tanning ability, greater freckling, and in adults lower mole nevus counts and a higher risk of melanoma and keratinocyte skin cancers (Duffy et al., 2010, Han et al., 2008, Han et al., 2011, Sulem et al., 2007, Zhang et al., 2013). The same high photoaging T allele has been shown to reduce activity of an *IRF4* enhancer in melanocytes (Praetorius et al., 2013). The direction of effect for the T allele of rs12203592 on nevus count and melanoma risk may be reversed in adolescents, with the T allele associated with lower rates of melanoma and higher mole counts (Duffy et al., 2010, Kvaskoff et al., 2011). We do not see evidence of a reversal in the BG6 association, as rs12203592's direction of effect is consistent in adolescents and in adults.

Of particular interest is the overlap in genes associated with two different phenotypes, skin patterning and pigmented spots/lesions (Jacob et al., 2015a). Both studies report the same

GWAS of Skin Aging

functional SNP in *IRF4*, rs12203592. The top BG6 SNP at *MC1R* is rs4268748, while for pigmented lesions it is rs35063026, and rs35063026 is also associated with BG6 ($p = 3.5 \times 10^{-10}$; Supplementary Table 1). While the LD between *MC1R* SNPs rs4268748 and rs35063026 is modest ($r^2 = 0.22$; 1000 genomes phase 1 v3), rs35063026 is in LD with the red hair allele rs1805007 ($r^2 = 0.83$), and conditioning on red hair alleles at *MC1R* abolished the association of rs35063026 with pigmented lesions (Jacobset al., 2015a). The minor allele of rs4268748 is modestly in LD with the minor red hair allele of both rs1805007 ($r^2 = 0.24$) and rs1805008 ($r^2 = 0.17$), likely explaining why it shows the strongest association with BG6. Conditioning on rs4268748 essentially abolishes the BG6 association of rs1805007 (Supplementary table 2; $p = 0.03$) and rs1805008 ($p = 0.4$). In summary, in this study the low skin pigmentation minor alleles of functional SNPs in *IRF4* and *MC1R* are associated with higher BG6 scores (more severe photoaging). The same alleles are associated with a greater percentage of the face covered with pigmented lesions (Jacobset al., 2015a).

In Jacobs *et al.*, (2015a) the pigmented spots/lesions measured included solar lentigines (SL) and seborrheic keratoses. SL are pigmented lesions that contain increased numbers of melanocytes as well as increased pigmentation of basal keratinocytes, and are a consequence of chronic UVR damage to the epidermis (Cario-Andre et al., 2004, Praetorius et al., 2014). Seborrheic keratoses are common benign skin tumours appearing with age, usually pigmented, and the evidence that they are associated with sun exposure and UVR damage is less conclusive (Hafner and Vogt, 2008). Furthermore, seborrheic keratoses are seen in all skin types while SL are more common in fairer skin. Skin patterning, as measured by BG6, and epidermal dyspigmentation including SL, are influenced by both chronological aging and photodamage. While the sites of the tests in the

GWAS of Skin Aging

two studies differ, both are assessing an outcome of sun damage (and natural aging) on a highly sun exposed site, and thus we find overlapping genes that mediate UVR protection (*MC1R*, *IRF4*). It is worth noting that, in the case of pigmented lesions, this association is at least partly independent of skin color (Jacobset al., 2015a), and the relationship between skin color and SL is complex (Praetoriuset al., 2014), so non-pigmentary roles of these genes may also be involved (e.g. (Mitraet al., 2012)).

In conclusion, we have identified a (to our knowledge previously unreported) genome-wide significant association with *SLC45A2*, and confirmed that functional SNPs in *IRF4* and *MC1R* are associated with BG6, a measure of wrinkling and photoaging. At each locus it is the lower pigmentation alleles that are associated with more severe photoaging. Low pigmentation alleles of *SLC45A2* and *MC1R* have been extensively associated with increased risk for melanoma and keratinocyte skin cancers (Bastiaenset al., 2001, Chatzinasiou et al., 2011, Han et al., 2006). *MC1R* red hair alleles, even in the heterozygous state, are associated with a higher burden of somatic mutations in melanoma (Robles-Espinoza et al., 2016). Photoaging is itself associated with keratinocyte skin cancers. Thus while photoaging, and general changes in appearance as we age, are of interest from a quality of life and cosmetic point of view, the genes discovered through BG6, which can be measured easily and quantitatively in large scale populations, may improve our understanding of cancer pathogenesis.

MATERIALS AND METHODS

SAMPLES

Three independent cohorts were used for this analysis. For the first, adolescent twins and their siblings were recruited in Brisbane, Australia, as a part of the Brisbane Twin Nevus Study (McGregor et al., 1999, Zhu et al., 1999, Zhu et al., 2007), totalling 3,759 adolescent twins and their siblings from 1,565 families; these were genotyped in two phases of 2,555 and 1,194 (Table 1). An unrelated set of adult twins comprising a total of 382 people from 197 families were drawn from an alcohol usage study (Heath et al., 1997, Whitfield et al., 2000) collected as a part of the same cohort. The second cohort, the Twins Eyes Study in Tasmania (TEST (Mackey et al., 2009)) contributed phenotype and genotype data from 136 twins. For the third cohort, 820 Raine participants were drawn from the 22-year follow-up (the Raine Eye Health Study, conducted between March 2012 and July 2014) of the ongoing prospective Western Australian Pregnancy Cohort Study funded by the Raine Medical Research Foundation (Raine) (Straker et al., 2015).

PHENOTYPE COLLECTION

For all participants in all cohorts, imprints of the back of the left hand were made using Affinis light-body silicone elastomer manufactured by Coltène AG, Altstätten, Switzerland (Barnes, 1973, Sarkany, 1962, Sarkany and Caron, 1965). Imprints for adolescent twins were made close to their 12th, 14th and 16th birthdays; age 12 BG6 scores were used preferentially, with missing data supplemented with age 14 data over age 16 as trait heritability decreases with age.

Coincident with the skin measurements, a sun exposure questionnaire was administered for 2,204

GWAS of Skin Aging

of the phase 1 adolescent twins (Supplementary Methods) (Shekaret al., 2005, Zhu et al., 1999).

Imprints for non-twin siblings were collected during their twin sibling's 12th birthday visit.

Imprints for the adult sample were collected at a single visit (Shekar et al., 2006, Shekaret al., 2005), as were the Raine and TEST sets.

A single rater (T. Luong) used a low power dissection microscope to rate all skin imprints according to the six categories of the Beagley–Gibson rating system with higher scores reflecting greater disruption of the normal pattern of the lines on the epidermis (Holman et al., 1984). The test-retest correlation for 50 BG6 measurements rescored a second time after nine months was 0.87 (95% CI 0.66-0.97) (Shekaret al., 2006).

ETHICAL STATEMENT

Approval to undertake this study was obtained from the Human Research Ethics Committee of the QIMR Berghofer Medical Research Institute. The Raine Study and TEST protocols were approved by the Human Research Ethics Committee of the University of Western Australia and the University of Tasmania, respectively. All studies conformed to the Declaration of Helsinki protocols, with participants giving their written informed consent.

GENOTYPING AND IMPUTATION

Adolescent samples were genotyped in two phases. Adolescent Phase 1, and TEST samples, were genotyped using Illumina Human610-Quad v1_B arrays at deCODE Genetics, Iceland. For

GWAS of Skin Aging

imputation they were merged with a larger set of individuals genotyped on Human610-Quadv1_B, and Human660W-Quad_v1_C arrays, and filtered to a common set of 482,586 markers post QC. Adolescent Phase 2 samples were genotyped on HumanOmniExpress-12v1-1_A, HumanOmni25M-8v1-1_B, and HumanCoreExome 12v1-0_C arrays from Illumina at the Diamantina Institute, University of Queensland, and filtered to a common set of 233,546 SNPs that passed QC in all sets.

Adult samples were genotyped on 317K, HumanCNV370-Quadv3_C, Human610-Quadv1_B, and Human660W-Quad_v1_C arrays by the Center for Inherited Disease Research, the University of Helsinki, and deCODE. For imputation they were merged with a large set of individuals genotyped on the same chips, and filtered to a common set of 276,755 SNPs post QC.

Adolescent, Adult, and TEST SNP data were excluded if they failed standard SNP filters in GenomeStudio as recommended by Illumina, and further cleaned out if Illumina BeadStudio GenCall < 0.7, MAF < 1%, HWE $P < 10^{-6}$, or call rate < 95%. X chromosome SNPs were filtered if their heterozygosity was >1% in males. Samples with missingness $\geq 5\%$ were dropped and principal component analysis in Eigenstrat (Price et al., 2006) was used to exclude samples with a PC1/PC2 value $> \pm 6SD$ from HapMap European populations. Identity by descent (IBD) estimates in PLINK were used to confirm pedigree structure. As the analysis for these four sets were done in Merlin-offline which explicitly models familial relationships, samples were not filtered for relatedness.

GWAS of Skin Aging

Adolescent, Adult, and TEST samples were then imputed to the 2010/11/23 v3 release of the 1000 Genomes Phase 1 using minimac (2013-07-17 version) with markers with ≤ 1 copy of the minor alleles removed. Imputation was performed in two phases based on chip overlap – (1) Adolescent Phase 1; (2) Adult; Adolescent Phase 2, and TEST; post imputation the four sets were analysed separately. For genotyped SNPs original genotype calls were used, for imputed SNPs imputation dosage scores were used.

Raine participants were genotyped on the Illumina 660 Quad Array at the Centre for Applied Genomics (Toronto, Ontario, Canada). As part of quality control (QC), individuals were excluded if they had IBD $\pi > 0.1875$ with another participant or $> 3\%$ missingness. SNPs were filtered out at HWE $p > 5.7 \times 10^{-7}$, missingness $> 5\%$ or a minor allele frequency < 0.01 . Potential population stratification was controlled for via a principal component analysis (PCA) using EIGENSTRAT (Price et al., 2006). Imputation of 22 autosomes was performed using MaCH v 2.3.0 software (Li et al., 2010) and the November 23, 2010 version of the 1000 Genome Project phase 1 European reference panel (Genomes Project et al., 2010).

GENOME-WIDE ASSOCIATION SCANS

For the Adolescent Phases 1 and 2, Adult and TEST samples GWAS were performed separately using 6-point BG6 scores as a continuous variable and imputed dosage genotypes using the Merlin-offline software (<http://www.sph.umich.edu/csg/abecasis/Merlin/>) under an additive model that explicitly models relatedness and produces unbiased estimates with standard errors corrected for the sample size inflation due to familial relationship (Chen and Abecasis, 2007). Sex, age,

GWAS of Skin Aging

age^2 , $\text{sex} \times \text{age}$ and $\text{sex} \times \text{age}^2$ were included as covariates.

For Raine, a linear regression analysis was performed in R (R Core Team, 2014) using the ProbABEL package (Aulchenko et al., 2010). The model was adjusted for age, sex, age^2 , $\text{age} \times \text{sex}$, $\text{age}^2 \times \text{sex}$ and the five principal components that accounted for the population stratification.

POST IMPUTATION QC AND META-ANALYSIS

Imputation quality was assessed by the ratio of imputed genotype dosage variance to the variance of the allele frequency under HWE (MaCH \hat{r}^2) (Liet al., 2010). For Adolescent Phase 1 and 2, TEST and Adult sets, SNPs with a $\hat{r}^2 < 0.5$ or $\text{MAF} < 0.05$ in any set were excluded; for Raine, SNPs with imputation quality < 0.5 were excluded; retained SNPs were meta-analysed using PLINK version 1.9 (<https://www.cog-genomics.org/plink2>) (Chang et al., 2015), with variant beta coefficients weighted by their inverse variance. Between study heterogeneity was assessed using the I^2 value (DerSimonian and Laird, 1986).

GWAS of Skin Aging

CONFLICT OF INTEREST

The authors state no conflicts of interest.

GWAS of Skin Aging

ACKNOWLEDGMENTS

Please see the supplementary documents

REFERENCES

- Abecasis GR, Cherny SS, Cookson WO, Cardon LR. Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002;30(1):97-101.
- Aulchenko YS, Struchalin MV, van Duijn CM. ProbABEL package for genome-wide association analysis of imputed data. *BMC Bioinformatics* 2010;11:134.
- Barnes IE. Techniques for the replication of skin surfaces. A new method. *Br J Dermatol* 1973;89(3):277-83.
- Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ, et al. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. *Am J Hum Genet* 2001;68(4):884-94.
- Battistutta D, Pandeya N, Strutton GM, Fourtanier A, Tison S, Green AC. Skin surface topography grading is a valid measure of skin photoaging. *Photodermatol Photoimmunol Photomed* 2006;22(1):39-45.
- Beagley J, Gibson IM. Changes in skin condition in relation to degree of exposure to ultraviolet light. Perth: Western Australia Institute of Technology School of Biology; 1980.
- Bilac C, Sahin MT, Ozturkcan S. Chronic actinic damage of facial skin. *Clinics in dermatology* 2014;32(6):752-62.
- Box NF, Duffy DL, Irving RE, Russell A, Chen W, Griffyths LR, et al. Melanocortin-1 receptor genotype is a risk factor for basal and squamous cell carcinoma. *J Invest Dermatol* 2001;116(2):224-9.
- Cario-Andre M, Lepreux S, Pain C, Nizard C, Noblesse E, Taieb A. Perilesional vs. lesional skin changes in senile lentigo. *J Cutan Pathol* 2004;31(6):441-7.
- Chang AL, Atzmon G, Bergman A, Brugmann S, Atwood SX, Chang HY, et al. Identification of genes promoting skin youthfulness by genome-wide association study. *J Invest Dermatol* 2014;134(3):651-7.
- Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK: rising to the challenge of larger and richer datasets. *GigaScience* 2015;4:7.
- Chatzinasiou F, Lill CM, Kypreou K, Stefanaki I, Nicolaou V, Spyrou G, et al. Comprehensive field synopsis and systematic meta-analyses of genetic association studies in cutaneous melanoma. *J Natl Cancer Inst* 2011;103(16):1227-35.
- Chen WM, Abecasis GR. Family-based association tests for genomewide association scans. *Am J Hum Genet* 2007;81(5):913-26.
- Costin GE, Valencia JC, Vieira WD, Lamoreux ML, Hearing VJ. Tyrosinase processing and intracellular trafficking is disrupted in mouse primary melanocytes carrying the underwhite (uw) mutation. A model for oculocutaneous albinism (OCA) type 4. *J Cell Sci* 2003;116(Pt 15):3203-12.
- DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986;7(3):177-88.
- Dooley CM, Schwarz H, Mueller KP, Mongera A, Konantz M, Neuhauss SC, et al. Slc45a2 and V-ATPase are regulators of melanosomal pH homeostasis in zebrafish, providing a mechanism for human pigment evolution and disease. *Pigment Cell Melanoma Res* 2013;26(2):205-17.
- Duffy DL, Box NF, Chen W, Palmer JS, Montgomery GW, James MR, et al. Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. *Hum Mol Genet* 2004;13(4):447-61.
- Duffy DL, Iles MM, Glass D, Zhu G, Barrett JH, Hoiom V, et al. IRF4 variants have age-specific effects on nevus count and predispose to melanoma. *Am J Hum Genet* 2010;87(1):6-16.
- Fritschi L, Battistutta D, Strutton GM, Green A. A non-invasive measure of photoageing. *Int J Epidemiol* 1995;24(1):150-4.
- Gandini S, Sera F, Cattaruzza MS, Pasquini P, Zanetti R, Masini C, et al. Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic

GWAS of Skin Aging

- factors. *Eur J Cancer* 2005;41(14):2040-59.
- Genomes Project C, Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, et al. A map of human genome variation from population-scale sequencing. *Nature* 2010;467(7319):1061-73.
- Graf J, Hodgson R, van Daal A. Single nucleotide polymorphisms in the MATP gene are associated with normal human pigmentation variation. *Hum Mutat* 2005;25(3):278-84.
- Green AC. Premature ageing of the skin in a Queensland population. *Med J Aust* 1991;155(7):473-4, 7-8.
- Grundberg E, Small KS, Hedman AK, Nica AC, Buil A, Keildson S, et al. Mapping cis- and trans-regulatory effects across multiple tissues in twins. *Nat Genet* 2012;44(10):1084-9.
- Hafner C, Vogt T. Seborrheic keratosis. *J Dtsch Dermatol Ges* 2008;6(8):664-77.
- Han J, Kraft P, Colditz GA, Wong J, Hunter DJ. Melanocortin 1 receptor variants and skin cancer risk. *Int J Cancer* 2006;119(8):1976-84.
- Han J, Kraft P, Nan H, Guo Q, Chen C, Qureshi A, et al. A genome-wide association study identifies novel alleles associated with hair color and skin pigmentation. *PLoS Genet* 2008;4(5):e1000074.
- Han J, Qureshi AA, Nan H, Zhang J, Song Y, Guo Q, et al. A germline variant in the interferon regulatory factor 4 gene as a novel skin cancer risk locus. *Cancer Res* 2011;71(5):1533-9.
- Heath AC, Bucholz KK, Madden PA, Dinwiddie SH, Slutske WS, Bierut LJ, et al. Genetic and environmental contributions to alcohol dependence risk in a national twin sample: consistency of findings in women and men. *Psychol Med* 1997;27(6):1381-96.
- Holman CD, Armstrong BK, Evans PR, Lumsden GJ, Dallimore KJ, Meehan CJ, et al. Relationship of solar keratosis and history of skin cancer to objective measures of actinic skin damage. *Br J Dermatol* 1984;110(2):129-38.
- Hughes MC, Strutton GM, Fourtanier A, Green AC. Validation of skin surface microtopography as a measure of skin photoaging in a subtropical population aged 40 and over. *Photodermatol Photoimmunol Photomed* 2012;28(3):153-8.
- Hughes MC, Williams GM, Baker P, Green AC. Sunscreen and prevention of skin aging: a randomized trial. *Ann Intern Med* 2013;158(11):781-90.
- Jacobs LC, Hamer MA, Gunn DA, Deelen J, Lall JS, van Heemst D, et al. A Genome-Wide Association Study Identifies the Skin Color Genes IRF4, MC1R, ASIP, and BNC2 Influencing Facial Pigmented Spots. *J Invest Dermatol* 2015a;135(7):1735-42.
- Jacobs LC, Liu F, Pardo LM, Hofman A, Uitterlinden AG, Kayser M, et al. IRF4, MC1R and TYR genes are risk factors for actinic keratosis independent of skin color. *Hum Mol Genet* 2015b;24(11):3296-303.
- Kadekaro AL, Leachman S, Kavanagh RJ, Swope V, Cassidy P, Supp D, et al. Melanocortin 1 receptor genotype: an important determinant of the damage response of melanocytes to ultraviolet radiation. *FASEB J* 2010;24(10):3850-60.
- Kvaskoff M, Whiteman DC, Zhao ZZ, Montgomery GW, Martin NG, Hayward NK, et al. Polymorphisms in Nevus-Associated Genes MTAP, PLA2G6, and IRF4 and the Risk of Invasive Cutaneous Melanoma. *Twin Res Hum Genet* 2011;14(5):422-32.
- Laville V, Le Clerc S, Ezzedine K, Jdid R, Taing L, Labib T, et al. A Genome-Wide Association Study in Caucasian Women suggests the involvement of HLA genes in the severity of facial solar lentigines. *Pigment Cell Melanoma Res* 2016.
- Lavker RM, Kwong F, Kligman AM. Changes in skin surface patterns with age. *J Gerontol* 1980;35(3):348-54.
- Le Clerc S, Taing L, Ezzedine K, Latreille J, Delaneau O, Labib T, et al. A genome-wide association study in Caucasian women points out a putative role of the STXBP5L gene in facial photoaging. *J Invest Dermatol* 2013;133(4):929-35.
- Li Y, Willer CJ, Ding J, Scheet P, Abecasis GR. MaCH: using sequence and genotype data to

GWAS of Skin Aging

- estimate haplotypes and unobserved genotypes. *Genet Epidemiol* 2010;34(8):816-34.
- Liu F, Hamer Merel A, Deelen J, Lall Japal S, Jacobs L, van Heemst D, et al. The MC1R Gene and Youthful Looks. *Current Biology* 2016;26(May 9):1-8.
- Mackey DA, Mackinnon JR, Brown SA, Kearns LS, Ruddle JB, Sanfilippo PG, et al. Twins eye study in Tasmania (TEST): rationale and methodology to recruit and examine twins. *Twin Res Hum Genet* 2009;12(5):441-54.
- Maresca V, Flori E, Bellei B, Aspite N, Kovacs D, Picardo M. MC1R stimulation by alpha-MSH induces catalase and promotes its re-distribution to the cell periphery and dendrites. *Pigment Cell Melanoma Res* 2010;23(2):263-75.
- McGregor B, Pfitzner J, Zhu G, Grace M, Eldridge A, Pearson J, et al. Genetic and environmental contributions to size, color, shape, and other characteristics of melanocytic naevi in a sample of adolescent twins. *Genet Epidemiol* 1999;16(1):40-53.
- Mishra A, Macgregor S. VEGAS2: Software for More Flexible Gene-Based Testing. *Twin Res Hum Genet* 2015;18(1):86-91.
- Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. *Nature* 2012;491(7424):449-53.
- Pelle E, Mammone T, Maes D, Frenkel K. Keratinocytes act as a source of reactive oxygen species by transferring hydrogen peroxide to melanocytes. *J Invest Dermatol* 2005;124(4):793-7.
- Praetorius C, Grill C, Stacey SN, Metcalf AM, Gorkin DU, Robinson KC, et al. A polymorphism in IRF4 affects human pigmentation through a tyrosinase-dependent MITF/TFAP2A pathway. *Cell* 2013;155(5):1022-33.
- Praetorius C, Sturm RA, Steingrimsson E. Sun-induced freckling: ephelides and solar lentigines. *Pigment Cell Melanoma Res* 2014;27(3):339-50.
- Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet* 2006;38(8):904-9.
- R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria 2014.
- Robles-Espinoza CD, Roberts ND, Chen S, Leacy FP, Alexandrov LB, Pornputtapong N, et al. Germline MC1R status influences somatic mutation burden in melanoma. *Nature communications* 2016;7:12064.
- Sarkany I. A method for studying the microtopography of the skin. *Br J Dermatol* 1962;74:254-9.
- Sarkany I, Caron GA. Microtopography of the Human Skin. Studies with Metal-Shadowed Replicas from Plastic Impressions. *J Anat* 1965;99:359-64.
- Seddon JM, Egan KM, Zhang Y, Gelles EJ, Glynn RJ, Tucker CA, et al. Evaluation of skin microtopography as a measure of ultraviolet exposure. *Invest Ophthalmol Vis Sci* 1992;33(6):1903-8.
- Shekar SN, Duffy DL, Montgomery GW, Martin NG. A genome scan for epidermal skin pattern in adolescent twins reveals suggestive linkage on 12p13.31. *J Invest Dermatol* 2006;126(2):277-82.
- Shekar SN, Luciano M, Duffy DL, Martin NG. Genetic and environmental influences on skin pattern deterioration. *J Invest Dermatol* 2005;125(6):1119-29.
- Song X, Mosby N, Yang J, Xu A, Abdel-Malek Z, Kadarko AL. alpha-MSH activates immediate defense responses to UV-induced oxidative stress in human melanocytes. *Pigment Cell Melanoma Res* 2009;22(6):809-18.
- Straker LM, Hall GL, Mountain J, Howie EK, White E, McArdle N, et al. Rationale, design and methods for the 22 year follow-up of the Western Australian Pregnancy Cohort (Raine) Study. *BMC Public Health* 2015;15:663.
- Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Magnusson KP, et al. Genetic determinants of hair, eye and skin pigmentation in Europeans. *Nat Genet*

GWAS of Skin Aging

- 2007;39(12):1443-52.
- Westra HJ, Peters MJ, Esko T, Yaghootkar H, Schurmann C, Kettunen J, et al. Systematic identification of trans eQTLs as putative drivers of known disease associations. *Nat Genet* 2013;45(10):1238-43.
- Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, et al. Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000;66(4):1246-58.
- Zhang M, Song F, Liang L, Nan H, Zhang J, Liu H, et al. Genome-wide association studies identify several new loci associated with pigmentation traits and skin cancer risk in European Americans. *Hum Mol Genet* 2013;22(14):2948-59.
- Zhu G, Duffy DL, Eldridge A, Grace M, Mayne C, O'Gorman L, et al. A major quantitative-trait locus for mole density is linked to the familial melanoma gene CDKN2A: a maximum-likelihood combined linkage and association analysis in twins and their sibs. *Am J Hum Genet* 1999;65(2):483-92.
- Zhu G, Montgomery GW, James MR, Trent JM, Hayward NK, Martin NG, et al. A genome-wide scan for naevus count: linkage to CDKN2A and to other chromosome regions. *Eur J Hum Genet* 2007;15(1):94-102.

GWAS of Skin Aging

TABLES

Dataset	Adolescent		Adult	TEST	Raine
	Phase 1	Phase 2			
MZ	384 pairs	194 pairs	60 pairs	19 pairs	0
DZ	634 pairs	292 pairs	46 pairs	42 pairs	0
Singleton twins	110	55	170	8	0
Siblings	409	167	0	6	0
Unrelated	0	0	0	0	820
Total N	2,555	1,194	382	136	820
Total families	1,061	504	197	71	820
Mean Age (sd)	15.3 (1.9)	14.4 (2.0)	44.6 (9.8)	9.6 (3.0)	22.2 (0.6)
Female:male	1,304:1,251	649:545	228:154	69:67	412:408

Table 1 Population description

GWAS of Skin Aging

Hg19 Position	SNP	Gene	A1 effect allele	A2	A1 Freq.	min r^2	Fixed p	Fixed Beta	I^2
5:33952106	rs185146	<i>SLC45A2</i>	C	T	0.06	0.52	4.1×10^{-9}	-0.25	0
6: 396321	rs12203592	<i>IRF4</i>	A	G	0.21	0.90	8.8×10^{-13}	0.13	0 ^a
16:90026512	rs4268748	<i>MC1R</i>	C	T	0.29	0.95	1.2×10^{-15}	0.13	1.8
16:89986117	rs1805007	<i>MC1R</i>	T	C	0.09	0.83	1.2×10^{-10}	0.18	0
16:89986144	rs1805008	<i>MC1R</i>	T	C	0.08	0.98	1.1×10^{-5}	0.12	24.6

Table 2: Loci with SNPs with p -values $< 5 \times 10^{-8}$ following the fixed effects meta-analysis. We have reported the top SNP, and if known, additional functional SNPs at the same loci. The full results, including random effects estimates and individual GWAS data, for all SNPs with p -values $< 1 \times 10^{-5}$ can be found in Supplementary Table 1. Note Table 1 results are given for the minor allele for ease of interpretation; as indicated in Supplementary Table 1 effect sizes may be reported for the other allele. SNP positions are Hg19. Bold indicates likely functional gene; otherwise nearest gene is listed. A1 Freq is the frequency of allele 1 in the combined dataset, weighted by sample N. min r^2 is the minimum imputation quality score across contributing GWAS. Beta is the change in BG6 score for each copy of the A1 allele. A negative effect size indicates allele 1 is associated with reduced BG6 scores, and reduced skin aging.

GWAS of Skin Aging

I^2 is the measure of heterogeneity of effect sizes across the included studies, and a low I^2 score indicates that the signals are homogenous.^a:







